EXHIBIT A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 4741
TSANG et al.	Examiner: Ruth A. Davis
Application No.: 10/658,437	Technology Center/Art Unit: 1651
Filed: September 8, 2003	DECLARATION OF DR. WEN-GHIH TSANG UNDER 37 C.F.R. §1.132
For: CD56 POSITIVE HUMAN ADULT PANCREATIC ENDOCRINE PROGENITOR CELLS	
Customer No.: 20350	

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Wen-Ghih Tsang, Ph.D., being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

2. I am an inventor on this application and am currently Senior Vice President of Research and Development at AmCyte, Inc. I received a Ph.D. in Medicinal Chemistry from Boston College in 1978. I was a post-doctoral fellow at the Massachusetts Institute of Technology from 1978-1980, working in the fields of cell-surface chemistry and applied enzymology. From 1980-1982 I was a research chemist with the Shell Oil Company. I began to work on cell encapsulation and transplantation while at Damon Biotech, Inc. (renamed

Abbott Biotech, Inc.) from 1982 to 1990. While at Damon I progressed from Senior Scientist to the position of Vice President of Development and Scientific Fellow. I continued to work on cell transplantation at Diacrin, Inc. from 1990 to 1995 as Director of Process Development and then as Director of Clinical Manufacturing and Quality Control. Since 1995 I have continued to work on cell encapsulation and transplantation at Amcyte, Inc. I am an author on a number of research papers and am an inventor on a number of patents. A copy of my curriculum vitae is attached hereto as Exhibit B and includes a list of publications and patents.

3. The present invention is a method of obtaining a culture of propagating pancreatic cells that exhibit a CD56 protein as a cell surface marker. The method includes the steps of isolating pancreatic cells from a pancreas, seeding the pancreatic cells in a culture vessel to induce expression of the CD56 protein on the cell surface; and harvesting the pancreatic cells from the culture vessel. Pancreatic cells that specifically bind to the CD56 binding reagent are separated from pancreatic cells that do not bind to the CD56 binding reagent to culture of propagating pancreatic cells that exhibit a CD56 protein as a cell surface marker. Because they are able to divide, the propagating pancreatic cells are undifferentiated or dedifferentiated cells.

4. I have read and am familiar with the contents of this patent application. In addition, I have read an Office Action, dated September 5, 2006, received in the present case and references cited in the Office Action. It is my understanding that the Examiner first alleges that the claimed invention is obvious over Fung *et al.* (US Patent No. 6,326,201) in view of Shipley *et al.* (1997).

5. According to the Examiner, Fung *et al.* teaches a method of obtaining a culture of pancreatic cells by obtaining cells from human pancreas, selecting cells by FACS or panning, and differentiating cells into aggregates of insulin producing cells. The Examiner concedes that Fung *et al.* does not teach use of the CD 56 antibody to select cells. The Examiner alleges that Shipley *et al.* teaches that CD56 is expressed in pancreatic islet cells, a differentiated cell type. The Examiner further alleges that one of ordinary skill would have been motivated by the teaching of Shipley *et al.* to use the CD56 binding reagent in the method of Fung *et al.* to select for propagating pancreatic cells. This declaration provides evidence that expression of a

CD56 protein on a differentiated pancreatic islet cell as allegedly taught by Shipley *et al.* would not lead one of skill to expect expression of CD56 on an undifferentiated, propagating pancreatic cell. Therefore, one of skill would not have expected to successfully use a CD56 binding agent to isolate undifferentiated pancreatic cells with the methods disclosed in Fung *et al.*

6. Pancreatic islet cells are a differentiated cell type and do not undergo cell division. The CD56 pancreatic cells isolated in the claimed methods are dedifferentiated cells that are capable of cell division. Expression of a particular protein in a differentiated cell cannot be used to predict that the same protein will be expressed in a less differentiated cell from the same tissue. Multiple examples of changes in protein expression during cellular differentiation are known to those of skill. The first example is submitted as Example C: P. W. Kincade and J. M. Gimble, *B Lymphocytes, in* Fundamental Immunology, Third Edition 47-51 (W. E. Paul, ed., 1993). Figure 5 at page 49 of Kincade and Gimble shows marker expression as hematopoietic stem cells differentiate into mature B lymphocytes and plasma cells. Both the mature B lymphocytes and plasma cells express, *e.g.*, the CD23 protein. However, the earliest precursor cells, hematopoietic and lymphoid stem cells, do not express the CD23 protein. In fact, many of the markers expressed on differentiated B cells are not expressed on the undifferentiated precursor cells. Thus, those of skill would not expect a B lineage precursor cell to express a particular protein simply because it is expressed on a mature B lymphocyte or plasma cell.

7. Protein expression varies as pancreatic cells mature and differentiate. An example of this variation in protein expression is submitted as Exhibit D: Schwitzgebel *et al.*, *Development* 127:3533-3542 (2000). Schwitzgebel *et al.* disclose expression patterns of the neurogenin 3 protein during differentiation of pancreatic islet cells. Neurogenin 3 is expressed in islet cell precursors. *See, e.g.*, Schwitzgebel *et al.* at page 3534, right column. However, neurogenin was not co-expressed with markers of fully differentiated islet cells, and therefore is not expressed in fully differentiated islet cells. *See, e.g.*, Schwitzgebel *et al.* at paragraph bridging pages 3536 and 3537. This result demonstrates that expression of a particular protein in an undifferentiated pancreatic cell cannot be predicted by the protein expression pattern found in a differentiated pancreatic cell.

8. Based only on the knowledge that a particular protein is expressed in a differentiated cell, those of skill would not reasonably expect to find that protein expressed in an undifferentiated propagating cell. Therefore, based only on the knowledge that a particular protein, *e.g.*, CD56, is expressed in a differentiated pancreatic cell, those of skill would not reasonably expect to isolate a population of propagating pancreatic cells using an agent that specifically binds to the protein expressed on a differentiated pancreatic cell.

9. This declaration also provides evidence that the harsh methods used by Shipley *et al.* to retrieve a CD56 epitope for staining, could not be combined with the methods of Fung *et al.* to obtain a culture of propagating pancreatic cells that exhibit the CD56 protein as a cell surface marker.

10. The claimed methods include steps of isolating pancreatic cells from a pancreas, seeding the pancreatic cells in a culture vessel, and culturing the cells to induce expression of the CD56 protein. Induction of CD56 expression after culture is disclosed in the specification at Example 6, page 47. To supplement that disclosure, Exhibit E is submitted. Slide 1 of Exhibit E shows flow cytometric analysis of cells from a human pancreas before and after disruption and culture. The top two panels show the cell profile before culture. The lower two panels show the cell profile after culture. The Y-axis measures CD56 expression (denoted SA11) and the X-axis measures expression of aldehyde dehydrogenase (ALDH), a housekeeping protein. Proteins were detected using labeled antibodies. The right panel uses an anti-CD56 antibody and the left panel uses an isotype control for the anti-CD56 antibody. Slide 2 provides numerical values for the results. Before culture, the pancreatic cells did not express CD56 at levels over the control. After culture, a large increase in CD56 expression is seen. Cell viability is required for this induction of CD56 expression.

11. Cell viability cannot be maintained using the methods described in Shipley *et al.* Shipley *et al.* determined CD56 levels in intact paraffin-embedded tissue using an anti-CD56 antibody. Shipley *et al.* state that they did not detect CD56 with antibody unless they first retrieved the CD56 epitope by microwaving the tissue. Shipley et al described microwave epitope retrieval as an **essential** step of their detection method. *See, e.g.*, Shipley *et al.* at page

91, right column. Using the retrieval method required by Shipley *et al.* on intact pancreas tissue would kill any living cells, making the step of culturing cells to induce CD56 expression ineffective. Shipley *et al.* also indicate that their detection method did not always give results that were in agreement with flow cytometry data. For example, Shipley *et al.* disclose that only 5 of 10 cancer cell samples that were positive for CD56 by FACS analysis were CD56 positive using the disclosed epitope retrieval method. *See, e.g.*, Shipley *et al.* at paragraph bridging pages 92 and 93. Thus, the methods of Shipley *et al.* cannot be simply combined with the methods of Fung *et al.* to isolate a culture of CD56 positive propagating pancreatic cells.

12. In view of the foregoing, it is my scientific opinion that expression of the CD56 protein on propagating pancreatic cells could not have been predicted based on the teachings of the cited references. It is also my scientific opinion that the teachings of the cited references cannot simply be combined to isolate a culture of CD56 positive propagating pancreatic cells.

Date: March 2, 2007 By: Wen-Ghih Tsang

Wen-Ghih Tsang, Ph.D.

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EXHIBIT B

Wen-Ghih Tsang, Ph.D. **Bio Sketch**

Senior Vice President, Research and Development

AmCyte, Inc.

With over twenty years of both research and management experience in the biotechnology industry, Dr. Tsang is a recognized expert in the field of cell therapy. Prior to joining AmCyte, Dr. Tsang was a senior leader at Diacrin Inc. where he successfully led scientific research in human adult stem cell culture as well as islet biology. He directed pioneering work in clinical development of cell and protein-based products. Working closely with the FDA, he achieved the approval to conduct two clinical trials for treating muscular dystrophy and Parkinson's disease. Dr. Tsang has been invited to speak in business and research meetings organized by institutions such as Marcus Evans, Imperial College London and professional societies.

As Director of Development at Abbott Biotech Inc. and Vice President and Scientific Fellow at Damon Biotech Inc., Dr. Tsang led the development of encapsulation technology with focused applications in islet transplantation and mammalian cell culture. These applications resulted in multi-million dollar manufacturing contracts and the establishment of a joint venture to develop islet transplantation for the treatment of diabetes. Dr. Tsang has been recognized by corporate awards and is the lead inventor of ten patents and many publications. Dr. Tsang conducted his post-doctorate research in cell culture at Massachusetts Institute of Technology, and received his Ph.D. in chemistry from Boston College.

Education

Massachusetts Institute of Technology 1978-1980
77 Massachusetts Avenue, Cambridge, MA 02139
Postdoctoral Associate with Professors. George M. Whitesides (Chemistry Department) and Howard Green (Biology Department)
Fields: Cell-Surface Chemistry and Applied Enzymology

Boston College 1974-1978
Chestnut Hill, Massachusetts 02167
Ph.D., Medicinal Chemistry (Natural Product Synthesis)
with Prof. T. Ross Kelly
Honors: Pass with Highest Distinction

Industrial Experience

AmCyte, Inc. 1995-Present
Sr. Vice President, Research and Development (2000-Present)
Chief Operating Officer (Acting) (1998-2000)
Vice President, Development (1995-1998)
Field: Encapsulation, Cell Biology, Clinical Transplantation

Diacrin, Inc. 1990-1995
Director, Clinical Manufacturing and Quality Control (1993-1995)
Director, Process Development (1990-1993)
Field: Cell Transplantation, Molecular Cell Biology and Immunology

Damon Biotech, Inc. (Renamed to Abbott Biotech, Inc. in 1990), 1982-1990
Vice President, Development & Scientific Fellow (1986-1990)
Director of Development, Biology & Chemistry (1985-1986)
Director of Development, Chemistry (1983-1985)
Sr. Scientist (1982-1983)
Fields: Polymer Chemistry, Microencapsulation, Mammalian Cell Culture, Controlled Release and Cell Transplantation

Shell Oil Company 1980-1982
Research Chemist, Corporate Research Department (1980-1982)
Field: Catalysis

Abstract of Industrial Experience

AmCyte Inc. (current)

Plan, establish, and oversee scientific programs from ground up. Key programs developed, both internally and with academic collaborations, range from basic research in cell biology and immune isolation to product development including IND submission and clinical trial.
Recruited by AmCyte to establish a business branch in Boston to tap into local talents and the resources of Tufts University Veterinary School. Promoted to company headquarters in Santa Monica to lead scientific programs as well as serving 2 years as acting Chief Operating Officer.

Diacrin, Inc.

Developed cellular transplantation technology, based on masking of MHC class I cell surface antigens, for the treatment of Parkinson's disease, muscular dystrophy and diabetes.

a. Neural Program (Parkinson's disease)

Developed a cutting edge xenotransplantation program for the implantation of porcine fetal ventral mesencephalon (VM) cells into Parkinson's patients. This program included the development of novel qualification procedures for tissue donors (pigs) as well as naval clinical manufacturing procedures for exnogeneic grafts. Filed position paper for tissue donor qualification and grafts drug mater file (DMF) with the FDA. This was the first xenotransplantation program received the FDA approval for human clinical trial.

b. Myoblast Program (Muscular Dystrophy)

Developed processes for cloning, phenotyping and culturing of pure human myoblasts. Also developed a injectable grade media formulation to maintain the viability of the myoblasts for more than 48 hours. This patented formulation enabled the shipment of living myoblasts to all major cities of the world. DMF filed in 1993. Clinical trials (for the treatment of Muscular Dystrophy) using this high quality myoblast product were conducted at Boston and San Francisco.

Developed using pure primary human myoblasts as vehicles for gene therapy. Myoblasts were transfected with beta-gal and human growth hormone genes. The expression levels of these transgenes were high and they were further increased by 2-3 fold following the induction of differentiation of these transfected myoblasts into myotubes. Initiated pre-clinical transplantation studies with transfected myoblasts/myotubes.

c. Diabetes Program

Developed an effective process for isolating islets of Langerhans (the insulin producing cells) from pig donors. Performed more than 150 successful isolations. Methods for culturing, testing and preserving of islets were carefully investigated.

d. Directed the production of clinical grade anti-SLA F(ab')2 fragment for immunomodulation of cells for transplantation.

e. Facility Design

Directed, from design to completion, the renovation of two buildings located in the historical Charlestown Navy Yard into research and production multiple use facility. The tissue processing suites were maintained as class 10,000 clean area for the clinical production of human myoblasts and porcine fetal neural cells.

Damon Biotech, Inc. (Abbott Biotech)

Developed biocompatible microencapsulation systems for tissue culture, controlled release and transplantation applications.

a. Polymer Chemistry (Microencapsulation)

Developed several series of homopolymers and copolymers of amino acids to form membranes over alginate gel spheres. These polymers generated membranes with permselectivity ranging between <20K MW and >700K MW.

Built a GLP polymer pilot laboratory where kilograms of polyamino acids were produced each month to make capsules for mammalian cell culture and other applications. Also produced polymers for pharmaceutical applications under business contract.

b. Mammalian Cell Culture

Developed mammalian cell culture systems, using microcapsules, for the production of monoclonal antibodies and other cell products (TPA, proUK, etc.).

Operated a large scale mammalian cell culture facility for 18 months, during this period, kilograms of cell products (MAb) were produced to fulfill production contracts.

c. Controlled Release

Developed a novel aqueous based controlled release delivery system for bovine somatotropin (bST; a labile 22K MW protein) under development contracts with

several major pharmaceutical companies. One formulation demonstrated effective bST release in cows for more than two weeks.

d. Transplantation of "Islets of Langerhans"

Microencapsulated islets were shown to survive longer both in vitro and in vivo, presumably due to the stabilization and immunoisolation provided by microcapsules. A licensing arrangement was made to commercialize this technology.

e. Developed budgets and managed up to 50 reports

Shell Development Company

Exploratory Catalysis

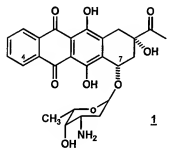
Developed new Ruthenium-Borane catalyst systems, involving zeolites, for he conversion of synthesis gas into chemicals. Four patents resulted from this work.

Abstract of Academic Experience

with Prof. T. Ross Kelly at Boston College

a. Synthesis of 4-Demethoxydaunomycin **1**

A new synthetic route to 4-demethoxy-7-deoxydaunomycinone **2** has been developed. The procedure affords **2** in 30% yield from 3 and involves a key Diels-Alder addition between quinizarinquinone **3** and the functionalized diene **4**.



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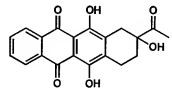

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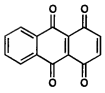

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b. Development of a Convenient Mercaptide Reagent "Lithium Thiomethoxide"

Lithium thiomethoxide, a stable off-white solid, was prepared and was demonstrated to be an effective reagent for the cleavage of hindered esters and epoxides and for the demethylation of methylaryl ethers.

with Professors George M. Whitesides (Chemistry) and Howard Green (Cell Biology) at MIT.

A multidisciplinary program to explore the science of cell–surface interactions

a. Cell-Surface Chemistry

Developed methods to chemically modify and physically coat the surface of polystyrene petri dishes. The surface properties of those dishes were characterized. Cell growth on these surfaces was investigated in order to probe the factors which control the attachment of anchorage-dependent mammalian cells. Developed methods to selectively break the cell-cell or cell-substrate bonds of cultured keratinocytes.

b. Applied Enzymology

The relation between the structure of a dithiol and its ability to prevent the autoxidation of proteins was studied as part of a project to utilize enzyme in organic synthesis.

Publications and Patents

1. "Studies Directed Toward the Synthesis of Anthracycline Antibiotics. A Regiospecific Diels-Alder Approach to Adriamycin and Related Aglycones" T.R. Kelly, R.N. Goerner, Jr., J.W. Gillard, W.G. Tsang, **Abstracts of the 172nd ACS National Meeting, August 1976, San Francisco.** This paper was subsequently reported in a full page article in Chemistry in Canada, December 8, 1976.

2. "Lithium Thiomethoxide: A Convenient Mercaptide Reagent" T.R. Kelly, H.M. Dali, and W.G. Tsang, **Tetrahedron Letters 44, 3859-3860 (1977).**

3. "The Synthesis of 4-Demethoxydaunomycin" T.R. Kelly and W.G. Tsang, **Tetrahedron Letters 46, 4457-4460 (1978).**

4. "Zeolite-Ruthenium-Borane Catalyst Composition" W.G. Tsang and L.H. Slaugh **U.S. Patent 4,405,499 (1983).**

5. "Syngas Conversion Process" W.G. Tsang and L.H. Slaugh **U.S. Patent 4,415,675 (1983).**

6. "Syngas Conversion Process" W.G. Tsang and L.H. Slaugh **U.S. Patent 4,415,676 (1983).**

7. "Zeolite-Ruthenium-Borane Catalyst Composition" W.G. Tsang and L.H. Slaugh **U.S. Patent 4,466,907 (1984).**

8. "An Efficient, Regiospecific Synthesis of (+)-Daunomycinone" T.R. Kelly, L. Ananthasubramanian, K. Korah, J.W. Gillard, R.N. Goerner Jr., P.F. King, J.M. Lyding, W.G. Tsang and J. Vaya **Tetrahedron 40 4569-4577 (1984).**

9. "Development of ENCAPCEL System for In Vivo Biomedical Applications" W.G. Tsang **Proceedings of the 12th International Symposium on Controlled Release of Bioactive Materials Geneva, Switzerland. 241-242 (1985).**

10. "Xenotransplantation of Microencapsulated Canine Islets into Diabetic Mice." R. Calafiore, N. Koh, F.L. Shienvold, W.G. Tsang, A. Shyr, and R. Alejandro, (intro. by D.H. Mintz) **Abstracts of Clinical Research. (1986)**

11. "Controlled Release of Insulin Microencapsulated by ENCAPCEL PROCESS" C. Bagul, A. Magee, A. Shyr, and W.G. Tsang. **Proceedings of the 13th International Symposium on Controlled Release of Bioactive Materials Norfolk, Virginia USA. 41-42 (1986).**

12. "Xenotransplantation of Microencapsulated Canine Islets into Diabetic Mice." R. Calafiore, N. Koh, F.L. Shienvold, W.G. Tsang, A. Shyr, R. Alejandro, and D.H.

Mintz **Abstract of Second Assisi International Symposium on Advanced Models for the Therapy of Insulin-Depended Diabetes. (1986)**

13. "Microencapsulation of Living Cells and Tissues. Theoretical and Practical Aspects" Calafiore R, Alejandro R, Tsang WG, Needell SD, and Mintz DH **Recenti. Prog. Med 78(2): 87-90 (1987)**

14. "Encapsulation of Materials" W.G. Tsang and A.W. Shyr. **U.S. Patent 4,663,286 (1987)**

15. "Capsule Loading Technique" W.G. Tsang **U.S. Patent 4,683,092 (1987)**

16. "Xenotransplantation of Microencapsulated Canine Islets in Diabetic Mice" Calafiore R, Calcinaro F, Basta G, Pietropaolo M, Falorni A, Dunnebacke R, Piermattei M, Alejandro R, and Tsang WG. **Minerva Endrocrinol 13(4): 287-92 (1988)**

17. "The Synthesis of Poly(L-Ornithine Hydrogenbromide)" J.P. Pease, W.G. Tsang, A.S. Magee, and D.B. Konopacki **Macromolecular Synthesis, Volume 11, 63-68 (1990)**

18. "Sustained Release of Encapsulated Molecules" W.G. Tsang and A.S. Magee **U.S. Patent 4,923,645 (1990)**

19. "Stabilization of Aqueous Based Hydrophobic Protein Solutions and Sustained Release Vehicle" W.G. Tsang, A.S. Magee, A.W. Shyr and J.P. Pease **U.S. Patent Application 302,410.**

20. "Sustained Release of Encapsulated Molecules" W.G. Tsang and A.S. Magee, **U.S. Patent 4,923,645 (1990)**

21. "Transplantation of pancreatic islets in diabetic nonhuman primates" Steele D, Wallstrom A, Bleier KJ, Tsang WG, Austen W, Auchincloss H, Chappel S **Transplant Proc.;26(6):3317-8 (1994)**

22. "Injectable Culture Medium for Maintaining Viability of Myoblast Cells" Agatha Zawadzka, Wen-Ghih Tsang, and Robert H Brown **U.S. Patent 5,543,316 (1996)**

23. "Molecular Mass Distribution of Sodium Alginate by High-performance Size-exclusion Chromatography" Sherry X. Ci, Tanya H. Huynh, Leslie W. Louie, Andrew Yang, Bridget J. Beals, Nilesh Ron, Wen-Ghih Tsang, Patrick Soon-Shiong, and Neil P. Desai **Journal of Chromatography A. 864 199-210 (1999)**

24. "In Vitro Generated Islet-Like-Clusters Exhibit Similar Cellular Architecture and Function to Cultured Encapsulated Islets. Wen-Ghih Tsang, Tianli Zheng, Yan

Ping Wang and Nataliya Bufius. **Diabetes Volume 53 Supplement 2 :A455, (2004)**

25. "Functional Characterization of Encapsulated Human Islet for Clinical Trial. W-G. Tsang, CC. Huntenburg, T. Zheng, A. Schaeffer, YP. Wang and K. Lim. **Rachmiel Levine Diabetes and Obesity Symposia (2004).**

26. "Recent Preclinical Studies for Prospective Phase I/II Safety Study of Encapsulated Isolated Primary Human Pancreatic Islet Transplantation with Low Dose Short Term Immune Suppression in Patients with Type I Diabetes Mellitus". W-G. Tsang, CC. Huntenburg, T. Zheng[1], A. Schaeffer, YP. Wang and K. Lim. **American Transplant Congress April (2004)**

27. "Culturing Pancreatic Stem Cells Having a Specified, Intermediate Stage of Development" Wen-Ghih Tsang, Tianli Zheng and Chang Jiang Huang **U.S. Patent 6,759,039 (2004)**

28. **"**Development of Encapsulated Human Islet: Effect of Material Selection on Product Performance" W.G. Tsang, C.C. Huntenburg, T. Zheng, A. Schaeffer, Y.P. Wang, K. Lim, E. Leoncio and P. Tram **U.S. Department of Health and Human Services Sponsored Workshop on Immunobarriers for Pancreatic Islet Transplantation. Washington D.C. March 29-30, (2004)**

29. "Activation of Akt1 in Adult Primary Human Pancreatic Cells Enhances the Survival and Proliferation of Endocrine Lineage Cells" Florio M, Pong E, Kelley L, Tsang WG, Francki, A **Keystone Symposium: Toward Understanding Islet Biology, Taos, NM, (2006)**

30. "Activation of Akt1 in Primary Human Pancreatic Cells Improves Survival and Proliferation of Endocrine lineage Cells" Monica Florio, Erick Pong, Laura Kelly, Wen G Tsang, Aleks Francki. **Diabetes Volume 55 Supplement 1:A354, (2006)**

31. "Expression of NCAM in Cultured Adult Human pancreatic Cells Delineates Mature Endocrine cells and Putative Progenitors" Aleks Franki, Zhi-Duan Zhong, wen Chen, Yan P Wang, Jinghua Tang, Erick Pong, Andrew Schaeffer, Wen G Tsang. **Diabetes Volume 55 Supplement 1:A360, (2006)**

32. Selection ofPancreatic Endocrine Lineage Cells Using Protein Synthesis Inhibitors Chen HJ, Tu Y, Wang YP, Stevens J, Francki A, Tsang WG. **4th Annual Meeting of the Int. Society for Stem Cell Res., Toronto, CAN, (2006)**

33. "Growth Factor-Mediated Activation of Akt1 in Adult Primary Human Pancreatic Cells Enhances the Selective Survival and Proliferation of Endocrine Lineage Cells" Monica Florio, Erik Pong, Laura Kelley, Wen-Ghih Tsang and Aleksandar Francki **(manuscript submitted for publication to Molecular Cell Endocrinology, 2006)**

34. "Generation of Functional Islet-Like Clusters after Monolayer Culture and Intracapsular Aggregation of Adult Human Pancreatic Islet Tissue" Wen-Ghih Tsang, Tianli Zheng, Yanping Wang, Jinghua Tang, Howard B. Rind, Aleksandar Francki, and Nataliya Bufius **(manuscript accepted for publication in Transplantation 2006)**

35. "In situ maturation of cultured pancreatic stem cells having a specified, intermediate stage of development" Wen-Ghih Tsang Tianli Zheng, Yanping Wang **US Patent 7,101,546 B2 (2006)**

Fundamental Immunology, Third Edition, edited by William E. Paul
Raven Press, Ltd., New York © 1993.

EXHIBIT C

CHAPTER 3

B Lymphocytes

Paul W. Kincade and Jeffrey M. Gimble

Cells of the immune system which are specialized to make antibodies are termed *B lymphocytes* to denote their origin in bone marrow of adult mammals or in the bursa of Fabricius in birds (1). Their importance to host defense is underscored in rare immunodeficiency diseases (Chapter 38). Without B cells, and the antibodies they make, one can only survive if given frequent gamma-globulin injections, ideally derived from someone being exposed to the same infectious agents. The antibody molecules found in gamma globulin are extremely versatile in terms of effector functions and can even confer specificity to nonlymphoid cells such as monocytes, which passively pick them up. The most unique feature of B lymphocytes is their preprogrammed ability to make an extraordinary number (more than 10^8) of antibodies with different combining sites. This is achieved by creative use of immunoglobulin gene segments, and further "fine tuning" of the antibodies occurs in some B cells by somatic mutation. Sequential and selective replacement of C-terminal portions of antibody molecules by B lymphocytes allows them to function as membrane-bound receptors or as soluble molecules with highly specialized biological functions.

P. W. Kincade and J. M. Gimble: Department of Immunobiology, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104.

This chapter is intended as an introductory overview of B cells, with an emphasis on their morphology, formation during fetal and adult life, and developmental relationships to other blood cells. Perhaps the most exciting aspect of B lymphocytes is their use as a model differentiation system, in which the temporal and selective expression of many genes has been documented. We will consider the tissues in which B lymphocytes are made *de novo* and describe the events which are now known to occur in them. Rapid progress is being made in the enrichment of multipotential stem cells and in resolving stages of differentiation which culminate in the formation of functional B cells. We are beginning to understand some of the regulatory mechanisms which govern blood cell production and see them in terms of positive and negative signals delivered by cytokines, the extracellular matrix, and adhesion molecules.

We hope to capture some of the excitement of the field while giving a fundamental background to these versatile cells and their relationship to human disease. This is frequently best illustrated with selected examples of basic experiments and clinical observations. Our developmental perspective should lead to an appreciation of the heterogeneity of B lymphocytes and be helpful in understanding their involvement in malignancies. Subsequent chapters will provide more detailed considerations of the structure and products of immunoglobulin genes, as well

tors, followed by positive selection by electronic cell sorting for rare cells with other characteristics. In appropriate strains of mice, expression of small amounts of the Thy-1 and Ly-6a antigens has been an important criterion for stem cell purification (23,24) (reviewed in ref. 25).

The most appropriate and stringent criteria for definition of stem cells have been debated for some time (21). Much of the early work on these critical cells depended on use of the *in vivo* spleen colony-forming cell (CFU-s) assay (26). In this system, foci of proliferating hemopoietic cells appear in the spleens of irradiated mice after injection with limiting numbers of hemopoietic cells (26). Some of the colonies, and especially those arising late after transplantation, could arise from true multipotential stem cells. However, it is now clear that many of the foci develop from committed progenitors which have relatively restricted capacity for self-renewal and which may not be able to give rise to lymphocytes. It has now been possible to demonstrate that stem cell populations highly enriched by the methods described above can clearly self-renew and repopulate all blood cell lineages after transplantation to mice given lethal doses of irradiation. Furthermore, it has recently been demonstrated that single micromanipulated cells can proliferate in culture and give rise to precursors of B lymphocytes as well as cells in several other lineages (27). The most highly purified stem cell populations may still be heterogeneous, with a more quiescent, highly self-renewing population being distinguished by the lack of expression of a multidrug resistance gene (28). This property influences uptake of the fluorescent dye rhodamine 123 and provides an additional parameter for cell sorting (29).

Stem-Cell–Progenitor Relationships

Although the existence of stem cells is now certain and their properties are being actively studied, questions remain about their relationships to committed progenitors of the various lineages (Fig. 2). We will now consider various cloning procedures that have been important for investigating these issues (30–32). Cells destined to become erythrocytes proliferate and synthesize hemoglobin under the influence of erythropoietin. These have been designated "burst-forming units" or "colony-forming units"—erythrocyte (BFU-e or CFU-e). Colony-forming units for granulocytes and macrophages (CFU-c) can similarly be identified when cultured with "colony-stimulating factors" (G-CSF, G/M-CSF, M-CSF, etc.). The molecular structure of many such substances which influence formation of the various blood cells has been recently determined by means of recombinant DNA technology (33,34) (see below). Discovery of interleukin 7 made it possible to develop an *in vitro* cloning assay specific for the precursors of murine B lymphocytes (35). Cloning procedures which employ stromal cell monolayers have been used to detect even earlier progenitors which have not yet rearranged Ig genes (36).

Certain *in vitro* cloning conditions favor the formation of multiple blood cell types from a single presumptive stem cell. For example, "CFU-GEMM" can give rise to large colonies containing granulocytes, erythrocytes, macrophages, and megakaryocytes (30). Each of these cell types can also be present in splenic foci that appear in the CFU-s assay. However, it is not totally clear how stem cells enumerated by this assay, or by any of the cloning procedures now available, relate to early progenitors of lymphocytes. More abundant cells, which have some lymphocyte lineage features, can be distinguished experimentally from the majority of CFU-c and CFU-s (10). For example, cells capable of giving rise to B cells after transplantation can be selectively depleted, or enriched, with monoclonal antibodies to surface antigens (37,38). Furthermore, numbers of early B-cell precursors and CFU-c do not correlate during embryonic life. These observations indicate that cells destined to become myeloid or lymphoid diverge at some point during development as indicated in Fig. 2.

A distinct type of "lymphoid stem cell" which can give rise to B or T lymphocytes has never been formally identified. Both types of lymphocyte are absent in certain combined immunodeficiencies (Chapter 35); in addition to the similarities in morphology, both rearrange antigen receptor genes. Such shared features invite speculation that lymphoid stem cells might exist as an intermediate stage between multipotential stem cells and lymphocytes (39).

The early derivation of natural killer (NK) cells, which have many lymphocyte properties, is similarly unresolved. Neither immunoglobulin nor T-cell antigen-receptor genes rearrange in NK cells (40,41). Furthermore, NK cells are distinctive in their developmental dependence on bone marrow. Ablation of bone marrow by injection of mice with ^{89}Sr prevents NK cell formation, whereas other lymphocytes are still produced (42,43). Mutant mice with severe combined immunodeficiency disease (SCID) have intact NK cells and myeloid cells, but no B or T lymphocytes (44). Finally, injection of monoclonal antibodies to a cell-surface antigen can be used to selectively deplete NK cells (45). Thus, NK cells could arise via a specialized lineage as indicated in Fig. 2.

Resolution of B-Lineage Progenitors

There is something of a continuum as stem cells lose the option of forming other types of blood cells and progress through a series of committed progenitor stages to eventually yield functional B lymphocytes. These stages have been operationally defined and distinguished to dif-

FIG. 3. Cell-surface immunoglobulin is evenly distributed on B cells (*arrows*) seen by phase contrast microscopy (**A**). When cross-linked with bivalent rhodamine-labeled anti-immunoglobulin antibodies, it is aggregated in small clusters. When the cells are metabolically active, this is swept into a "cap" at one pole of the cell, termed the *uropod* (red fluorescence illumination in panel **B**). Four T lymphocytes are stained in this preparation with a fluorescein-labeled antibody to Thy-1 (green illumination in panel **C**).

ferent degrees in experimental animals and in humans. Accordingly, there is less than total agreement on the number that can be resolved and what they should be called. Lymphocytes are only called "B cells" if they synthesize and express surface immunoglobulin (sIg) molecules (Fig. 3). We will discuss specialized "memory" and "B1" categories of B cells in a later section. Their immediate precursors are generally referred to as "pre-B cells," and the most widely accepted characteristics are the expression of μ heavy chains, which are detectable in the cytoplasm and the absence of sIg (Fig. 4). Large, actively proliferating pre-B cells can clearly be resolved from small ones, and one might also distinguish a very transient and late stage of pre-B cells which bear some μ heavy chains in association with a "surrogate" light-chain complex (see below). The term "pro-B cell" has come into use to denote cells which, on the basis of surface marker expression, are clearly in the B lineage, but do not yet synthesize any Ig chains. Early, intermediate, and late stages of pro-B cells have been resolved in mice on the basis of size and selective gene expression. The reader may wish to compare recent reviews on this issue

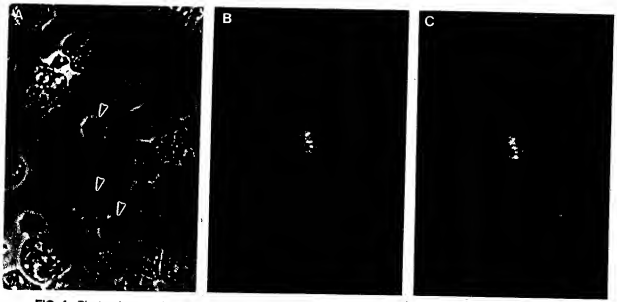

FIG. 4. Photomicrographs of murine fetal liver cells stained for surface immunoglobulin with fluorescein-labeled antibodies to immunoglobulin and then placed on a slide. After being permeabilized by fixation, the cells were again stained with rhodamine-labeled anti-μ. The central cell identified by phase contrast microscopy (**A**) is also visible with green (**B**) and red (**C**) fluorescence filters. It is therefore a B cell. Faint staining for cytoplasmic, but not surface, immunoglobulin is typical of the large and small pre-B cells arrowed in the lower part of the figure. (Photomicrographs courtesy of Dr. Max D. Cooper.)

which relate to experimental animals (46,47) and humans (48). A simplified version of this process is illustrated in Fig. 5.

Stem cells first develop in the extraembryonic yolk sac, as well as within intraembryonic tissues. Although cells in both sites are probably multipotential, lymphocytes most likely derive from cells within the embryo (49–52). In mammals, hemopoietic stem cells are most abundant in embryonic spleen and liver, which are the principal sites of hemopoiesis until birth. Cells bearing markers of early lymphoid progenitors emerge in those tissues (approximately 8 weeks of gestation in human fetal liver), followed by pre-B cells and then sIg-bearing B cells (16). In experimental circumstances, the first B cells to emerge can be functionally competent, and, in humans, intrauterine infection can result in some antibody formation before birth. However, the composite of markers which typify mature B lymphocytes in adults is not completely expressed in embryos, and those early cells differ in other ways from newly formed B cells found in adult bone marrow. Thus the initial process and events associated with B-cell formation during ontogeny are not identical to the adult steady-state production of lymphocytes which continues throughout life (see discussion of B1 cells below.) From shortly after birth, the majority of all blood cells except T lymphocytes are made within bone marrow. This was established in experiments where radiolabeled thymidine was locally infused to isolated limbs of guinea pigs or mice (17,18,53). Lymphocytes in marrow of the injected bone steadily accumulated the label, and this revealed that they were locally made from actively proliferating precursors. Most of the newly formed lymphocytes were shown to be B cells when it became possible to distinguish them on the basis of Ig expression (54). We will now discuss how the restricted expression of many proteins in cells of this lineage has been discovered and how these can be used as markers to follow progression of differentiating cells.

"Alloantibodies" can be prepared by immunizing one inbred strain of mice with lymphocytes from another (55). This approach provided the first well-characterized experimental reagents for distinguishing cells. For example, the Thy-1 marker is preferentially expressed on T cells in the mouse, and anti-Thy-1 antibodies can be used in the presence of complement to prepare T-cell-depleted cell suspensions for studies of B-cell function. "Congenic" inbred strains differing only for a genetic polymorphism affecting the Thy-1 antigen were developed, and the same approach was used to define many other lymphocyte markers in the mouse (56). These were usually named Ly-1, Ly-2, etc., or Lyt-1, Lyt-2, Lyb-2, etc., to indicate that the antigens were found preferentially on T or B lymphocytes.

With development of the monoclonal antibody tech-

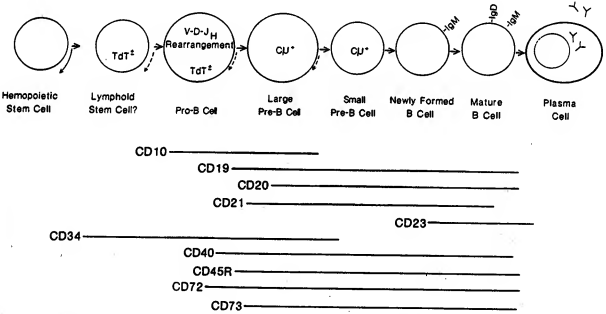

FIG. 5. Some of the major stages of human B-lymphocyte formation are shown with changes in size and gene expression that have been used to distinguish them. The enzyme terminal deoxynucleotidyl transferase (TdT) appears before rearrangement and expression of Ig heavy-chain genes. The μ chains are detectable in the cytoplasm of pre-B cells before complete IgM molecules appear on the surface at the immature B-cell stage. Acquisition and/or loss of certain membrane glycoproteins can be detected with monoclonal antibodies. Some of these are indicated with their "cluster of differentiation" (CD) numbers and discussed in the text.

nique (see Chapter 12), it became possible to prepare well-defined and homogeneous antibodies to antigens expressed on outbred individuals, including humans (57). A confusing nomenclature arose because of the development of multiple antibodies in different laboratories to the same, or very similar, glycoproteins. This problem was largely resolved at international workshops, where it became possible to group similar reagents which appeared to detect related antigens (58). These are now organized with a "cluster of differentiation" (CD) nomenclature, and a partial listing of those expressed on human B cells is illustrated in Fig. 5 (48,58–60). Further definition of cell-surface protein antigens has come from recombinant DNA technology. In several cases it became clear that when the amino acid sequence was deduced, the CD antibodies to human markers recognized antigens comparable to the Ly markers originally discovered in the mouse (e.g., ref. 61).

There are many examples of glycoproteins which are expressed in the same tissue-specific manner on the lymphocytes of mice and humans. For example, the general structure and expression of the genes for Ig of B cells, the T-cell receptor for antigen, and the Ly-1/Leu-1 (CD5) marker have been conserved through evolution. The common leukocyte antigen family (CD45) represents a particularly complex example of differential gene expression. Three exons of a single large structural gene encode up to five different-sized glycoproteins, or "isoforms," which are differentially expressed on virtually all blood cells except erythrocytes. B cells in mice, rats, and humans preferentially express the 220,000-dalton isoform of CD45 (62–67).

Some antigens are structurally similar in many species, but the cellular representation, or timing of acquisition of these markers on lymphocytes, can be quite different. The Thy-1 antigen is present in the brain of mice, rats, and humans, but it is a useful marker for mature T cells only in mice. Cells of the B-lymphocyte lineage in rats express large amounts of Thy-1, whereas very sensitive techniques are required to detect it on early lymphoid precursors and stem cells in mice (24,68,69).

A very important group of molecules on B cells are termed "major histocompatibility complex (MHC) class II antigens." These are 63,000-dalton heterodimers consisting of an alpha and beta chain in mice and humans. They bind antigen fragments, and the resulting complex is recognized on the B-cell surface by helper T cells. This close interaction is critical for one type of highly efficient and antigen-specific antibody response (Chapters 14 and 17). Class II molecules are similarly distributed on murine and human macrophages, dendritic cells, and Langerhans cells, and also function there as a recognition unit for T cells. However, while these vital structures are fully expressed on pre-B cells of humans, they are acquired in high density only at the B-cell stage of mice (70).

Most of the markers listed in Fig. 5 have been implicated in certain functions. Molecular cloning revealed that CD10 is a neutral endopeptidase, which was first described in rats (71). It is expressed on bone marrow stromal cells of mice and humans, but is readily detectable on human, but not murine, pre-B cells (72; C. Paige, *personal communication*). CD19 is a transmembrane glycoprotein which can noncovalently associate with components of the antigen receptor complex on B lymphocytes (73). Its total restriction to the B lineage makes it a very useful marker, and it presumably is essential for normal lymphocyte responses. Similarly, structural studies with CD20 suggest that it may be an ion channel, which could also function during receptor-mediated events (74). CD34 has recently been cloned and found to have a mucin-like structure (75). This marker has been frequently exploited to enrich early hemopoietic progenitors in humans and could be speculated to be a ligand for lectin-like adhesion molecules. Experiments using antibodies to mimic the natural ligand of CD40 indicate that it may be important for survival and replication of certain types of B cells (60). Particular isoforms of CD45 are relatively restricted to the B lineage, and phosphatase activity mediated by the cytoplasmic domain is thought to be critical to normal lymphocyte responses to foreign antigens (76).

Other molecules have properties which invite speculation about their requirement in immune responses. CD21 has a low, but measurable, affinity for the complement component C3d, and it is the receptor for Epstein–Barr virus, the pathogenic agent for infectious mononucleosis and Burkitt's lymphoma (77). Likewise, CD23 can act as a receptor for the Fc portion of the IgE class of immunoglobulins. The substrate specificity and expression of the enzyme ecto 5′-nucleotidase (CD73) on B cells is well known, but it remains to be determined how lymphocytes utilize it. Like Thy-1, this marker is anchored to the membrane via a glycosyl-phosphatidylinositol linkage (78,79). The function of many other molecules detectable on B cells is not yet clear. This is sometimes the case even when the primary structure, carbohydrate modification, cellular representation, time of acquisition, and chromosomal location of the structural gene are known.

It is important to emphasize that no one marker alone should be used to assign cells to, or position them within, a lineage with respect to stage of maturity. Studies using large numbers of monoclonal antibodies and tumor cells reveal that most markers are not absolutely restricted to a single cell type. Immunoglobulins represent one of few molecules which are only made by B lymphocytes, and their construction requires the coordinate expression of multiple genes (e.g., those for recombinase enzymes, heavy and light chains). There is reason to believe that during B-cell formation, all cells at an equivalent stage of maturity do not have to express the same molecules, or

at least the same amounts of the same markers (80). That is, although mature B cells require a number of receptors and other glycoproteins for proper immune function, there is no obvious reason that these have to be acquired in a set order. Expression of certain surface antigens is strikingly influenced by proximity of the cells to lymphokines, and others are affected by the age of the animal (e.g., some markers which typify B cells in adults are absent from embryonic B cells). For these reasons, tumor cells are currently classified as belonging to a particular stage of the B lineage by means of a "composite" of markers, no one of which is totally distinctive (81,82).

Some distinctive features of B-lymphocyte lineage cells cannot be appreciated by analysis of surface glycoproteins. As discussed below, no other lineage undergoes the complete sequence of immunoglobulin gene rearrangements, and there are other nuclear and cytoplasmic events which characterize them. The enzyme terminal deoxynucleotidyl transferase (TdT) is present in the nucleus of early progenitors of B and T lymphocytes (83,84). It catalyzes the random incorporation of nucleotides into exposed ends of DNA and is responsible for insertion of non-germ-line ("N") residues in rearranging immunoglobulin genes (85,86). The importance of TdT may be in contributing to the diversity of antibody combining sites (Chapter 10). This has been a useful marker for distinguishing lymphocyte progenitors at a relatively early stage in mice and humans. Other enzymes associated with the Ig gene rearrangement process are also expressed in a transient fashion, but antibodies have not been described which would permit the timing to be accurately determined (87).

POPULATION DYNAMICS

The only practical estimates of the rates of production and survival times of lymphocytes come from studies of experimental animal models. However, these findings presumably have value when extrapolated to humans. Several generalizations should be made before briefly considering the approaches that have been used and conclusions which result from them. First, most of the available data are statistical. That is, numbers of cells generated in a particular compartment per hour, or transiting a particular stage of maturation, are averages. It is not certain that every newly formed B cell is made via the resulting predicted sequence, or in that particular time frame. Second, it is important to remember that B cells can be made in two ways. Not only do they emerge from the bursa of Fabricius and bone marrow, but they are also made by self-renewal (i.e., by division of existing B cells). Finally, "life span" can have two meanings. An individual B cell can die, or it can give rise to more B cells by dividing. In the latter case, an incorporated radiolabel would be diluted out such that the cell seemed to disappear. Actually, the clone it represented would have survived in expanded form. We would like to know if potentially dangerous clones of autoreactive B cells are continuously emerging throughout life, and at what rate; the magnitude of total daily B-cell formation; how many newly formed cells are intrinsically defective, and if they can be rescued; the potential survival time of really useful B cells whose antibody specificities are currently protective; and how the life spans and migration streams of memory cells differ from those of virgin lymphocytes. Brief answers to some of these questions will be attempted here, while more detailed information can be found in subsequent chapters.

For understanding the kinetics of lymphocyte formation, no techniques have been more valuable than those utilizing radiolabeled thymidine, and this is especially the case when combined with cell-surface and cytoplasmic markers (reviewed in ref. 18). When administered to mice as a single dose, label is rapidly incorporated into large B-lineage cells, including some large pre-B cells (88). At intervals thereafter, approximately half as much radioactivity appears in small pre-B cells, and, finally, small B cells emerge with the same quantity of radiolabel. These findings suggest that large pre-B cells divide once, or very few times, before shrinkage, and there is little or no subsequent proliferation during the time it takes for light-chain genes to be expressed (Fig. 6). Molecular analysis of large B-lineage cells (at the late "pro-B" stage) suggests that most have rearranged the heavy-chain, but not the light-chain, genes (89,90). The latter event then presumably occurs during a nonmitotic period, which has been estimated to average 2 days. Careful inspection of kinetic data obtained by thymidine incorporation, and with a metaphase arrest technique, indicated that a young mouse makes around 5×10^7 B cells per day (91). This would translate to around 150 billion in a human, an intermediate value between the estimated production of erythrocytes and neutrophils (92).

The spleen of a mouse contains approximately 7.5×10^7 B cells, so unless some of the product of bone marrow quickly dies, or is intrinsically defective, the entire peripheral population would be rapidly replaced. Considering the errors associated with rearrangements that lead to incorrect reading frames and so on, as many as half of all newly formed B cells could be defective (93). The kinetic data are *consistent* with twice as many pre-B cells as B cells being made (91), so some loss could occur at that stage. However, a sequential rearrangement mechanism has been discovered through which nonfunctional (or functional) variable-region gene segments can be replaced by other V regions from an upstream position (94). It is not known if this is normally used as a salvage procedure within bone marrow. The fate of defective B cells is thus unclear, but special "quality control" mechanisms may have evolved to recognize and eliminate them (95,96). Dying lymphocytes are ultimately ingested and destroyed by macrophages (97,98).

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Expression of neurogenin3 reveals an islet cell precursor population in the pancreas

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SUMMARY

Differentiation of early gut endoderm cells into the endocrine cells forming the pancreatic islets of Langerhans depends on a cascade of gene activation events controlled by transcription factors including the basic helix-loop-helix (bHLH) proteins. To delineate this cascade, we began by establishing the position of neurogenin3, a bHLH factor found in the pancreas during fetal development. We detect neurogenin3 immunoreactivity transiently in scattered ductal cells in the fetal mouse pancreas, peaking at embryonic day 15.5. Although not detected in cells expressing islet hormones or the islet transcription factors Isl1, Brn4, Pax6 or PDX1, neurogenin3 is detected along with early islet differentiation factors Nkx6.1 and Nkx2.2, establishing that it is expressed in immature cells in the islet lineage. Analysis of transcription factor-deficient mice demonstrates that neurogenin3 expression is not dependent on neuroD1/BETA2, Mash1, Nkx2.2, Nkx6.1, or Pax6. Furthermore, early expression of neurogenin3 under control of the *Pdx1* promoter is alone sufficient to drive early and ectopic differentiation of islet cells, a capability shared by the pancreatic bHLH factor, neuroD1/BETA2, but not by the muscle bHLH factor, MyoD. However, the islet cells produced in these transgenic experiments are overwhelmingly α cells, suggesting that factors other than the bHLH factors are required to deviate from a default α cell fate. These data support a model in which neurogenin3 acts upstream of other islet differentiation factors, initiating the differentiation of endocrine cells, but switching off prior to final differentiation. The ability to uniquely identify islet cell precursors by neurogenin3 expression allows us to determine the position of other islet transcription factors in the differentiation cascade and to propose a map for the islet cell differentiation pathway.

Key words: Islet of Langerhans, Pancreas, Basic helix-loop-helix protein, neurogenin3, Mouse

INTRODUCTION

The pancreas forms from dorsal and ventral buds that appear at the foregut-midgut junction starting at embryonic day (E)9.5 in the mouse. Over the subsequent 10 days of fetal development, these two apparently uniform clusters of epithelial cells differentiate into the cell types that compose the adult pancreas: the endocrine cells of the islets of Langerhans, the duct cells, and the exocrine cells (for reviews see Sander et al. (1997); Slack (1995)).

The first detectable differentiated cells, starting at E9.5, are glucagon-expressing α cells followed sequentially by other islet cells: insulin-producing β cells, somatostatin-producing δ cells and pancreatic polypeptide-producing PP cells (Gittes and Rutter, 1992; Herrera et al., 1991; Pictet et al., 1972; Rall et al., 1973; Teitelman et al., 1993; Upchurch et al., 1994). Based on this order of appearance, a variety of transgenic experiments, and evidence that cells expressing more than one endocrine hormone are present early in pancreatic development, several models have been proposed to explain the developmental lineage of endocrine cells (Alpert et al., 1988; Herrera et al., 1994, 1991; Teitelman et al., 1993; Upchurch et al., 1994). These models have in common the supposition that the endocrine cells formed early in pancreatic development function as progenitors of the mature islet cells found later. This assumption has never been proved by direct lineage tracing.

Alternatively, a population of undifferentiated precursor cells may persist, allowing for new islet cell formation throughout development. After E13, when ducts can first be distinguished, new endocrine cells appear adjacent to, or even embedded in the ducts (Githens, 1988; Herrera et al., 1991;

Pictet et al., 1972; Teitelman and Lee, 1987), suggesting that either the duct cells themselves or some population of cells harbored among the duct cells may function as precursors for islet cells throughout development.

The morphologic changes that occur as endocrine cells differentiate from precursors depend on sequential changes in gene expression. Recent studies have focussed on the underlying molecular events that control these changes in gene expression. Gene disruption experiments in mice have demonstrated the importance of several transcription factors of the homeodomain family in islet cell differentiation. These include Pax6 (Sander et al., 1997; St-Onge et al., 1997) and Isl1 (Ahlgren et al., 1997), which are required for the normal formation of all islet cell types, Pax4, which is involved in the differentiation of β and δ cells (Sosa-Pineda et al., 1997), and Nkx6.1, which is required specifically for β cell formation (M. Sander and M. German, unpublished data). The absence of Nkx2.2 causes a reduction in α and PP cells, but also causes a specific block in late steps of β cell differentiation (Sussel et al., 1998).

Cell-type-specific (class B) members of the basic helix-loop-helix (bHLH) family of transcription factors play essential roles in the development and maintenance of many differentiated cell types. neuroD1/BETA2 is a class B bHLH factor expressed in the pancreas and involved in islet cell development and insulin gene transcription (Naya et al., 1995). Although the targeted disruption of the neuroD1/BETA2 gene in mice leads to a reduction in the endocrine cell mass at birth, this is largely due to an increase in apoptosis: endocrine cell formation is not blocked and insulin production continues in the remaining β cells (Naya et al., 1997). The persistence of islet cell genesis suggests that other bHLH proteins also may contribute to islet cell differentiation during the development of the pancreas.

The developing pancreas expresses a second, related bHLH protein, neurogenin3 (Sommer et al., 1996b). Based on experiments showing that early expression of neurogenin3 can cause early differentiation of islet cells, and evidence that neurogenin3 is not expressed in mature islet cells, Apelqvist et al. (1999) have concluded that neurogenin3 is expressed in islet cell progenitors and functions as a pro-endocrine gene driving islet cell differentiation. However, other bHLH factors, such as neuroD1/BETA2 were not tested in a similar fashion. Since many class B bHLH factors can drive terminal differentiation of a variety of cell types when expressed in the appropriate precursors (Arnold and Winter, 1998; Jan and Jan, 1993; Lee, 1997; Sommer et al., 1996a), and it was not established whether the cells normally expressing neurogenin3 are in the islet cell lineage, it remains uncertain whether neurogenin3 is the bHLH factor that normally functions as a pro-endocrine gene in the developing pancreas. Furthermore, it is not clear from these experiments whether neurogenin3 is sufficient to drive the differentiation of all four islet cell types, since only glucagon expression was examined.

Neurogenin3 is necessary, however, for the formation of all four islet cell types, since mice carrying a homozygous targeted disruption of the neurogenin3 gene lack any islet cells (Gradwohl et al., 2000). In the early pancreatic bud, neurogenin3 mRNA can be detected by in situ hybridization in proliferating cells expressing the homeodomain protein Pdx1, but not in hormone-expressing cells, while neuroD1 mRNA is detected in a non-replicating cell population that includes mature hormone-expressing cells (Jensen et al., 2000). Together, these results have led to the hypothesis that neurogenin3 lies upstream of neuroD1 in the pathway of islet cell differentiation, a conclusion that is supported by recent evidence that neurogenin3 can activate the neuroD1 promoter (Huang et al., 2000).

In the present study, we clarify the position of neurogenin3 in the hierarchy of islet transcription factors. We demonstrate by degenerate RT-PCR and in situ hybridization that islet cells and the developing pancreas express a broad group of class B bHLH genes, among the most abundant being neurogenin3 (*ngn3*), *neuroD1/BETA2*, *Mash1* and *neuroD4/Math3*. Immunohistochemical analysis reveals that neurogenin3 is indeed co-expressed with some markers of differentiating islet cells, including the β cell differentiation factor Nkx6.1, thus establishing that neurogenin3 marks islet cell precursors. In an experiment similar to that of Apelqvist et al. (1999), we confirm that neurogenin3 can drive the early differentiation of islet cells, but also find that neuroD1/BETA2 shares this capacity. However, these transgenic experiments only produce glucagon-expressing α cells. Thus other signals in addition to the pro-endocrine bHLH factors are required to develop the full spectrum of islet cell types. Together with earlier studies, these results provide the framework for a more detailed map of the differentiation pathway for pancreatic islet cells.

MATERIALS AND METHODS

RT-PCR

Total cellular RNA was extracted from rat islets and mRNA was prepared from αTC, β-TC3, mPAC and 266 cells using the RNaqueous extraction kit (Ambion) and DNase I treatment to remove contaminating genomic DNA. Oligo(dT)-primed reverse transcription of RNA was carried out for 1 hour at 42°C using Superscript II (Gibco). First strand cDNA served as a template for PCR amplification as previously described (Sommer et al., 1996b) using Taq polymerase (Fisher) and the following degenerate primers derived from the helix 1 and the loop of the conserved bHLH encoding region of the neural class B bHLH genes (5′ primer: cgggatccAAT/CGA/CIC/AGIGAA/GC/AGIAAT/CC/AGIA/GT, 3′ primer: cggaattcAG/AIGTT/CTCIAT/CT/CTTIG/CA/TIAT/G/AT/CTT). PCR was carried out in a DNA Thermal Cycler (Perkin-Elmer) for eight cycles with a denaturation step at 94°C for 1 minute, annealing at 43°C for 1 minute and extension at 72°C for 1 minute, followed by an additional 40 cycles with the annealing temperature raised to 55°C. The Taq polymerase was added at 94°C during the first cycle. The 127 bp PCR product was purified from a NuSieve agarose gel by Quiaquick (Quiagen), subjected to a second round of PCR (40 cycles of 1 minute at 90°C, 1 minute at 5°C, and 1 minute at 72°C), digested with *Eco*RI and *Bam*HI, and cloned into the pBlueScript (KS)+ vector (Stratagene). In total, 107 clones were characterized and identified by sequencing with the Sanger dideoxy method.

In situ hybridization

For RNA in situ hybridization analysis of paraffin sections (5 μm), embryos and pancreases were processed, sectioned and hybridized with digoxigenin-labeled riboprobes as described by Neubuser et al. (1995) with some modifications. Digoxigenin-labeled sense and antisense riboprobes were detected with alkaline phosphatase-coupled anti-digoxigenin antibodies using BM purple (Boehringer Mannheim) as color substrate. Riboprobes used in this study were as follows: ngn1

(Ma et al., 1996), ngn2 and ngn3 (Sommer et al., 1996b), neuroD1 (Lee et al., 1995), and Mash1 (Guillemot et al., 1993).

To obtain a neuroD4/Math3 probe (Takebayashi et al., 1997), a λ-ZAP (Stratagene) cDNA library prepared from the mouse insulinoma cell line β-TC3 was screened by hybridization with the PCR product encoding neuroD4. From positive clones, a full length neuroD4 cDNA was selected and confirmed by sequencing with the Sanger dideoxy method.

Immunohistochemical and immunofluorescence assays

Immunohistochemistry and immunofluorescence assays were performed on paraffin sections as described previously (Sander et al., 1997). Primary antibodies employed in these assays: mouse anti-insulin antibody (Sigma) diluted 1:10000; guinea pig anti-insulin 1:10000 (Linco, St. Charles, MI); mouse anti-glucagon 1:10000 (Sigma); guinea pig anti-glucagon 1:10000 (Linco); guinea pig anti-peptide YY (kindly provided by G. Aponte (Upchurch et al., 1994)) 1:800; rabbit anti-Pax6-QNR antiserum 11 (kindly provided by S. Saule, Institut Pasteur, Lille, France) directed against the paired domain of quail PAX6 (Turque et al., 1994), diluted 1:4000; mouse anti-Isl1 (Developmental Hybridoma Bank) 1:200; monoclonal mouse anti-Nkx2.2 (kindly provided by T. Jessel) 1:50; rabbit anti-Nkx6.1 (Sussel et al., 1998) 1:6000; rabbit anti-Brn4 (kindly provided by M. G. Rosenfeld (Schonemann et al., 1995) 1:100; monoclonal mouse anti-skeletal muscle myosin heavy chain clone MF20 (Developmental Hybridoma Bank) 1:100; monoclonal mouse anti-smooth muscle actin clone 1A4, cy3-conjugated (Sigma) 1:200; mouse anti-PCNA and Ki-67 (Pharmigen) 1:4000. Secondary antibodies were used as described previously (Sander et al., 1997).

Neurogenin3 antigen was produced by inserting the coding sequence for the amino-terminal 95 amino acids from mouse neurogenin3 downstream of the glutathione S-transferase coding sequence in the pGEX-2T vector (Pharmacia). The resulting fusion protein was purified from *E. coli* and injected into rabbits and guinea pigs. 1:5000 (rabbit) or 1:4000 (guinea pig) dilutions were used for staining, and preimmune sera from both animals gave no staining at the same concentrations. Pdx1 antigen was produced by inserting the coding sequence for the carboxy-terminal 80 amino acids from mouse IPF1 downstream of the glutathione S-transferase coding sequence in the pGEX-2T vector (Pharmacia). The resulting fusion protein was purified from *E. coli* and injected into rabbits and guinea pigs. 1:4000 dilutions of the guinea pig antiserum were used for staining, and preimmune sera gave no staining at the same concentrations.

For immunofluorescence assays, Cy3- (Jackson ImmunoResearch Laboratories), Texas Red- and FITC-conjugated (Cappel) goat anti-rabbit, anti-guinea pig and anti-mouse were applied at a 1:200 dilution. Fluorescence was visualized with a Zeiss axioscope and a Leica confocal microscope.

Transgenic mice

The *Pdx1* promoter vector pBAT.PD17 was constructed by inserting the mouse *Pdx1* promoter (a 4.4 kb *XbaI-SmaI* fragment from the mouse *Pdx1* gene containing the transcription start site and promoter (Apelqvist et al., 1997; Wu et al., 1997)) and the human β-globin gene first intron upstream of the pBAT polylinker (German et al., 1992) and the SV40 late gene polyadenylation signal. A 663 bp DNA fragment encoding full length mouse *ngn3* cDNA was obtained by PCR from the mouse genomic neurogenin3 clone (Sommer et al., 1996b) and inserted into the pBAT.PD17 polylinker. The *neuroD1/BETA2* vector was constructed by cloning into pBAT.PD17 a 1.7 kb DNA fragment encoding the full length mouse cDNA (Lee et al., 1995) extending from the start codon through the 3′ UTR. The *MyoD* vector was constructed by cloning into pBAT.PD17 a 1 kb DNA fragment encoding the full length mouse cDNA (Davis et al., 1987) extending from the start codon through the first 63 bp of the 3′ UTR.

The vectors were linearized and purified, and transgenic mice were generated by pronuclear injection (1.5 ng/μl) into F_1 hybrid oocytes from C3Fe/B6 parents as described by Hogan et al. (1994). Genotypes were determined by PCR analysis of genomic DNA from tail biopsies. The primers used were: 5′ TGGAGAACTGTCAAAGCGATCTG (*Pdx1* primer for 5′) and 5′ CACATGCCCAGTTTCTATTGGTC (human β-globin intron for 3′).

Embryos were harvested at E12.5 or E18.5. A total of 6 *Pdx1-ngn3* animals were examined at E12.5, and 10 at E18.5. A total of 5 *Pdx1-neuroD1* animals were examined at E12.5, and 10 at E18.5. As is common in independent transgenic founders and has been seen previously in transgenic animals produced with the *Pdx1* promoter (Apelqvist et al., 1997, 1999), these animals display a spectrum of phenotypes. At E18.5, pancreatic size varied in both groups of transgenics, from normal to the extreme micro-pancreas shown in Fig. 8. The extreme micro-pancreas was observed in two animals in each group. Less variation was observed at E12.5, when the majority of pancreases from both groups contained more glucagon-expressing cells than their littermates. Those phenotypes observed in multiple independent founders are reported. The liver in the *Pdx1-ngn3* transgenic animal in Fig. 8A appears reduced in size; this abnormality was not observed in other transgenics. The transgenic animals were not grossly abnormal in size. Only a single control *Pdx1-MyoD* transgenic was sectioned and stained at E18.5; it is shown in Fig. 8.

RESULTS

Expression of bHLH genes in pancreatic cell lines and islets

Expression of mRNA encoding class B bHLH proteins was detected using a non-quantitative method of RT-PCR with degenerate oligonucleotide primers derived from the conserved bHLH-encoding region of *NeuroD1/BETA2*-related genes (Ma et al., 1996). cDNA was prepared from purified adult rat islets and from four mouse cell lines: the β cell line β-TC3 (Efrat et al., 1988), the α cell line α-TC1.6 (Hamaguchi and Leiter, 1990), the pancreatic ductal cell line mPAC (Yoshida and Hanahan, 1994), and the pancreatic exocrine cell line 266 (Ornitz et al., 1985). The PCR products encode 9 distinct sequences including NeuroD1/BETA2 (Lee, 1997; Naya et al., 1995), Mash1 (Guillemot et al., 1993), NeuroD2 (McCormick et al., 1996), neuroD4/Math3 (Takebayashi et al., 1997), neurogenins (ngn)1, 2 and 3 (Gradwohl et al., 1996; Ma et al., 1996; Sommer et al., 1996b), Mist1 (Lemercier et al., 1997), and meso1/scleraxis (Blanar et al., 1995; Cserjesi et al., 1995)

Table 1. Degenerate PCR of islet and pancreatic cell line cDNA*

	Rat islets	βTC3	αTC1.6	mPAC	266
neuroD1/BETA2	3	6	6		1
neuroD2	2	2			
neuroD4/Math3	5	4	3	2	7
neurogenin1	4	6	2		1
neurogenin2/Math4A	2			1	
neurogenin3	4	4	7	4	
Mash1			1		
mist		2	2		3
meso1/scleraxis				1	
non-bHLH		2		3	11

*RT-PCR was performed using the degenerate oligonucleotides described in the Materials and Methods section with RNA purified from the cell types indicated. The products were subcloned and sequenced. Each number indicates how many copies of that cDNA were sequenced from the cells shown. All of the sequences that were obtained are included.

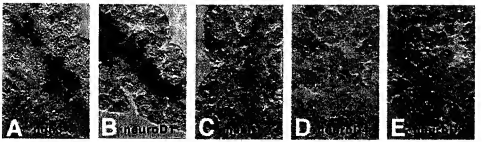

Fig. 1. Expression of bHLH genes at E15.5. In situ hybridization studies in E15.5 fetal mouse pancreas are shown for (A) *ngn3*, (B) *NeuroD1/BETA2*, (C) *Mash1*, (D) *NeuroD4* and (E) *NeuroD2*. 200× magnification. Only pancreatic tissue is shown.

(Table 1). Ngn3 and NeuroD4 were found in all the pancreatic endocrine cell lines and in rat islets; NeuroD4 was also found in the exocrine cell line. Only NeuroD1/BETA2, ngn3 and mist1 were previously known to be expressed in the pancreas (Apelqvist et al., 1999; Naya et al., 1997; Naya et al., 1995; Sommer et al., 1996b). Although degenerate PCR amplification does not produce quantitative information regarding expression level, these data provide a set of bHLH cDNAs from pancreatic cells for further analysis of expression levels and patterns.

Expression of bHLH factors in the pancreas in vivo

To determine the expression pattern in vivo of the bHLH factors detected by RT-PCR, in situ hybridization studies of the developing mouse pancreas were performed. Consistent with previous studies (Apelqvist et al., 1999; Sommer et al., 1996b), this analysis detects high expression levels of *ngn3* and *neuroD1* at E12.5 and E15.5 (Fig. 1 and data not shown). Low expression of *Mash1* and *neuroD4/Math3* can be detected at E15.5 (Fig. 1). At E12.5 neither *Mash1* nor *neuroD4/Math3* can be detected in the pancreas, although *Mash1* can be detected in scattered cells surrounding the pancreas (data not shown), probably due to expression in developing enteric neurons (Lo et al., 1991). Significant expression was not detected for neurogenins 1 and 2, or *neuroD2* at any date (data not shown).

Using polyclonal antisera, ngn3 can be detected as early as E11.5 in a few epithelial cells of the pancreatic bud, at E15.5 it reaches a peak of expression, decreases at E18.5, and is undetectable in the adult pancreas (Fig. 2 and data not shown). Although we did not detect ngn3 protein in the pancreas prior to E11.5, *ngn3* mRNA has been detected by in situ hybridization in scattered cells in the pancreatic bud as early as E9.5 (Apelqvist et al., 1999; Jensen et al., 2000). At E15.5 ngn3 is detected in the nuclei of scattered ductal cells and occasional periductal cells. Immunofluorescent double labeling at E15.5 shows no co-staining with several endocrine cell products, including insulin, glucagon, somatostatin, pancreatic polypeptide, islet amyloid polypeptide and peptide YY (Fig. 3 and data not shown), suggesting that if ngn3 plays a role in islet cell development, it is switched off prior to final differentiation.

Immunofluorescent double labeling was performed for ngn3 and two proteins expressed in islet cell precursors: Nkx6.1, a β cell homeodomain transcription factor (Jensen et al., 1996; Rudnick et al., 1994); and Nkx2.2, a homeodomain factor expressed in all α, β, and PP cells, and required for normal β cell differentiation (Sussel et al., 1998). At E15.5 Nkx6.1 is co-expressed in 30-50% of cells expressing ngn3, and Nkx2.2 is expressed in 70-80% of cells expressing ngn3 (Fig. 4). These data demonstrate that ngn3 expression marks precursor endocrine cells, and is absent from differentiated endocrine cells.

ngn3 is not co-expressed, however, with two homeodomain transcription factors normally present within the pancreatic epithelium only in fully differentiated islet cells: the LIM-homeodomain factor Isl1 (Fig. 4), which is required for the differentiation of all islet cells (Ahlgren et al., 1997); and the

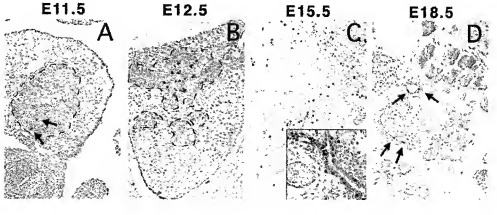

Fig. 2. Expression of neurogenin3 in the developing mouse pancreas. Immunohistochemical staining for neurogenin3 using rabbit anti-mouse neurogenin3 (1:5000) and peroxidase labeled goat anti-rabbit IgG (1:1000) in fetal mouse pancreas (A) at E11.5, (B) at E12.5, (C) at E15.5 and (D) at E18.5 (200× magnification). Arrows in A and D indicate nuclei expressing ngn3. Inset in C at 400× magnification shows nuclei staining for ngn3 in cells lining a duct.

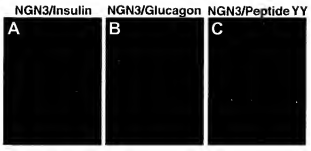

Fig. 3. Double immunofluorescence staining in fetal mouse pancreas at E15.5 with (A) with anti-insulin serum (cy3 label, red) and anti-ngn3 serum (fluorescein label, green); (B) anti-glucagon serum (cy3 label, red) and anti-ngn3 serum (fluorescein label, green), and (C) anti-peptide YY serum (cy3 label, red) and anti-ngn3 serum (fluorescein label, green). Photomicrographs imaged confocally. 400× magnification.

paired homeodomain factor Pax6 (data not shown), which is involved in islet formation and islet hormone synthesis (Sander et al., 1997; St-Onge et al., 1997). At E15.5 ngn3 is also not co-expressed with Brn4, a Pou-homeodomain transcription factor found in α cells (Hussain et al., 1997), or with Pdx1, a homeodomain transcription factor largely restricted to β and δ cells at this stage (Fig. 4). These results place these four factors late in the differentiation pathway.

Pancreatic expression of ngn3 in mice lacking islet transcription factors

To place ngn3 in the hierarchy of transcription factors involved in islet development, animals with targeted disruptions or mutations of several islet transcription factor genes were examined for ngn3 expression. Mice homozygous for a targeted disruption of the *NeuroD1/BETA2* or *Mash1* genes were examined at E18.5, and *Nkx6.1* and *Nkx2.2* null animals were examined at E15.5 and E18.5. In all of these animals, ngn3 is still expressed at levels equivalent to the levels in wild-type littermates (Fig. 5, and data not shown). ngn3 is also expressed in pancreases of embryos homozygous for the Pax6 mutant allele, Sey^{Neu} (loss of function mutation) (Fig. 5).

The pancreases of mice lacking *Mash1* were also examined for differences in islet development. At E18.5, no obvious abnormalities can be detected in islet morphology or the expression patterns of the islet hormones insulin, glucagon, somatostatin and PP (Fig. 6 and data not shown).

The expression levels of ngn3 in these mutant animals demonstrate that its expression is not dependent on any one of these transcription factors, and together with the co-staining data suggest a function upstream of Nkx6.1, Nkx2.2 and Pax6.

Transgenic expression of bHLH factors

To further investigate the function of ngn3, transgenic mice over-expressing ngn3 early during development were created using the *Pdx1* promoter to drive expression of a mouse *ngn3* cDNA in pancreatic progenitor cells. Pdx1 is a homeodomain transcription factor expressed in the gut endoderm near the foregut-midgut junction prior to formation of the pancreas (E8.5). As the gut matures, scattered expression of Pdx1 persists in the duodenum and antral stomach. When the dorsal and ventral pancreatic buds form (E9.5-E10.5), Pdx1 then is expressed broadly in the epithelial cells forming the pancreatic bud, but is inactivated in the few endocrine cells (mostly α cells with rare β cells) that differentiate at this stage. Starting around E13, Pdx1 expression becomes limited to β cells and δ cells (Guz et al., 1995; Offield et al., 1996; Ohlsson et al., 1993; Oster et al., 1998). The portion of the mouse *Pdx1* promoter used in these studies is sufficient to drive the expression of linked genes in the same pattern (Apelqvist et al., 1997; Gerrish et al., 2000; Wu et al., 1997).

Founder mice were analyzed at E12.5 and E18.5. Similar to a previous report (Apelqvist et al., 1999), at E12.5 in mice over-expressing ngn3, there is a marked increase in the number of glucagon-expressing cells in the pancreatic buds relative to wild-type litter mates, with most of the cells converted to an α cell fate (Fig. 7). Glucagon staining can also be detected in a few cells lining the duodenum near the pancreas (Fig. 7D) and the antral stomach in some transgenic animals indicating that expression of ngn3 in areas of Pdx1 expression outside the pancreas (Offield et al., 1996) can induce the development of ectopic endocrine cells.

Transgenic animals were examined for ectopic expression of the *ngn3* transgene, but none was detected. This result is not surprising since the *Pdx1* promoter driving the *ngn3* transgene

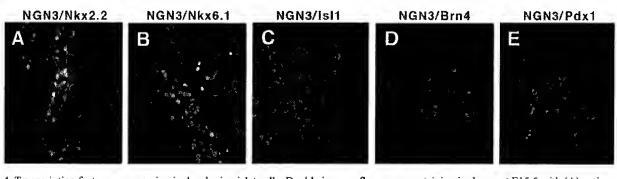

Fig. 4. Transcription factor co-expression in developing islet cells. Double immunofluorescence staining is shown at E15.5 with (A) anti-Nkx2.2 serum (cy3 label, orange) and anti-ngn3 serum (fluorescein label, green), (B) anti-Nkx6.1 serum (fluorescein label, green) and anti-ngn3 serum (cy3 label, orange), (C) anti-Isl1 serum (cy3 label, orange) and anti-ngn3 serum (fluorescein label, green), (D) anti-Brn4 serum (fluorescein label, green) and anti-ngn3 serum (cy3 label, orange), and (E) anti-Pdx1 serum (cy3 label, orange) and anti-ngn3 serum (fluorescein label, green). Photomicrographs in B,C, and D were confocally imaged. Note the yellow staining in the co-staining nuclei in A and B. 400× magnification.

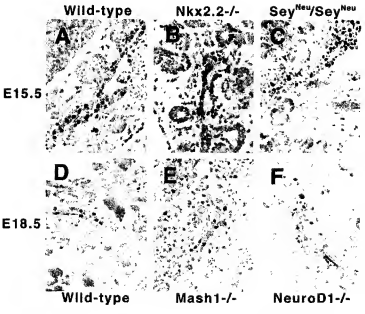

Fig. 5. Neurogenin3 expression in mutant mice. Immunohistochemical staining for neurogenin3 is shown at E15.5 (A-C) and E18.5 (D-F) in the pancreas of mice with the following transcription factor mutations: (A) *Nkx2.2*$^{+/+}$, (B) *Nkx2.2*$^{-/-}$, (C) *Sey*^{Neu}/*Sey*^{Neu} (*Pax6* null mutation), (D) *Mash1*$^{+/+}$, (E) *Mash1*$^{-/-}$, (F) *neuroD1/BETA2*$^{-/-}$ as positive control. 400× magnification. In each panel, neurogenin3 can be detected in the nuclei of cells scattered along a large duct within the pancreatic parenchyma.

is not active in α cells, the predominant cell type in these pancreases (Gerrish et al., 2000; Wu et al., 1997). We presume that *ngn3* is expressed in progenitor cells at an early stage (Offield et al., 1996), but is extinguished in the differentiated cells examined at E12.5. To test the possibility that the increase in α cells is due to increased proliferation, E12.5 transgenic pancreas was tested for the presence of markers for dividing cells, PCNA and Ki-67, by immunohistochemistry; the α cells did not express these proliferation markers (Fig. 7F and data not shown). Given the premature increase in α cells in conjunction with the depletion of undifferentiated precursor cells in the E12.5 pancreas, we conclude that the increase in the α cell population results from premature differentiation of pancreatic epithelial cells expressing the *ngn3* transgene into α cells.

By E18.5, transgenic animals show a dramatic decrease of the size of the pancreas (Fig. 8A), consistent with the depletion of precursors due to premature differentiation into a non-dividing population of mature cells. At E18.5 the fraction of endocrine cells is grossly increased, and this increase can be accounted for by an increase in the proportion of glucagon-expressing cells (Fig. 9). In addition, the glucagon-positive cells are no longer limited to the periphery of islets as in the wild-type littermates, but now fill out entire islets. Double immunofluorescence labeling for glucagon and insulin confirms the predominance of glucagon over insulin, but does not detect any cells expressing both hormones (Fig. 9I).

Transgenic mouse embryos expressing NeuroD1/BETA2 under control of the *Pdx1* promoter were also examined at E12.5 and E18.5. Like the *ngn3* transgenic animals, these animals demonstrate early differentiation of endocrine cells, with a marked reduction or absence of duct and exocrine structures (Figs 7C, 8B, 9E,F). Again the overwhelming majority of endocrine cells express glucagon.

Finally, to determine the specificity of the effects of ngn3 and NeuroD1/BETA2 on pancreas development, transgenic mice over-expressing *MyoD*, a class B bHLH gene normally expressed during muscle development and not normally expressed in the pancreas (Sassoon et al., 1989), were examined. In common with *ngn3* and *neuroD1/BETA2*, transgenic expression of *MyoD* results in a decrease in the size of the pancreas at E18.5; but there is a decrease in endocrine cells, especially insulin-expressing cells (Fig. 9G,H). In addition, *MyoD* expression results in the formation of strands of multinucleated cells that express the muscle-specific proteins, smooth muscle actin (a marker also found in embryonic skeletal muscle; Woodcock-Mitchell et al., 1988) and skeletal muscle myosin heavy chain (Fig. 9J and data not shown).

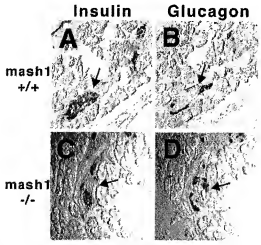

Fig. 6. Insulin and glucagon expression in mice lacking Mash1. Immunohistochemical staining is shown for insulin (A,C) and glucagon (B,D) in pancreases at E18.5 from Mash1$^{+/+}$ (A,B) and Mash1$^{-/-}$ (C,D) animals. (200× magnification). In each panel, the arrow indicates a forming islet, with insulin-expressing β cells in the center surrounded by glucagon-expressing α cells.

DISCUSSION

These studies demonstrate that the developing pancreas and mature islet cells express a variety of class B bHLH genes. Not all of these genes are expressed at substantial levels as gauged by in situ hybridization, nor are they all expressed at the same time. None the less, the proteins encoded by some of these genes, especially the multiple members of the neurogenin and neuroD family (Lee, 1997; Sommer et al., 1996b), are remarkably closely related; and their overlapping expression suggests a significant degree of redundancy.

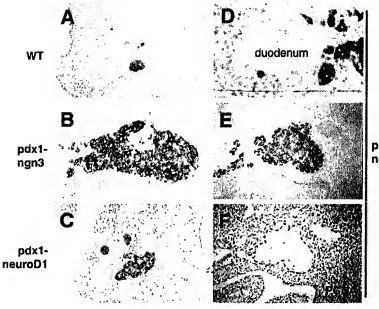

Fig. 7. Expression of islet hormones in transgenic mice at E12.5. Immunohistochemical staining is shown for glucagon in pancreases of a non-transgenic littermate (A) and transgenic fetuses expressing ngn3 (B) or neuroD1/BETA2 (C) (200× magnification). (D) Immunohistochemical staining for glucagon in the adjacent duodenum from the animal in B demonstrates scattered glucagon-expressing cells in the gut epithelium (400× magnification). (E,F) Serial sections of the pancreas and surrounding tissue from a transgenic fetus expressing ngn3 showing immunohistochemical staining for glucagon (E) and the proliferating cell nuclear antigen PCNA (F) (150× magnification). The glucagon expressing pancreatic epithelium in E is outlined with a dashed line that is superimposed on the serial section in F.

The presence of multiple bHLH factors in the pancreas is not surprising, since a similar degree of redundancy has been noted in the development and maintenance of other organ systems such as muscle or nervous system (Jan and Jan, 1993; Molkentin and Olson, 1996). This redundancy could explain the modest phenotype of mice lacking NeuroD1/BETA2 (Naya et al., 1997). However, although neurogenin3 is present in the *NeuroD1/BETA2* null animals, its expression is neither increased nor prolonged relative to the wild-type littermates, indicating that neurogenin3 does not compensate for the absence of neuroD1/BETA2.

Differences in the pattern and timing of expression of the bHLH genes in the developing pancreas suggests that their functions are not completely redundant, and they may have overlapping but distinct roles in the gene expression events controlling differentiation. The Mash genes are the mammalian homologues of the *Drosophila* proneuronal achaete-scute genes (Johnson et al., 1990), and in some examples Mash1 lies upstream of members of the neurogenin and neuroD gene families in the cascade of transcription factors controlling mammalian neural development (Cau et al., 1997; Lee, 1997). In the pancreas, however, Mash1 is not required either for expression of neurogenin3 or for endocrine differentiation. The absence of a pancreatic phenotype in animals lacking Mash1 could indicate the presence of functionally redundant factors at this step in pancreatic development, but its level of expression as indicated by in situ hybridization suggests that Mash1 may not be an important regulator of pancreatic endocrine cell differentiation.

Instead of Mash1, neurogenin3 or neuroD1 may be the furthest upstream members of the class B bHLH family and fill the role of the proneuronal genes in islet development. Members of both the neurogenin and neuroD families can drive vertebrate neurogenesis (Lee et al., 1995; Ma et al., 1996), and both neurogenin3 and neuroD1 can individually drive endocrine cell genesis. But which of these genes fills this role in vivo? The phenotype of animals lacking neuroD1 or Mash1 and its expression pattern suggest that neurogenin3 is the earliest pro-endocrine bHLH gene expressed. In contrast, animals lacking neurogenin3 fail to express neuroD1 (Gradwohl et al., 2000), suggesting that the role of neurogenin3 may be to activate neuroD1, which in turn directs endocrine cell differentiation, similar to the proposed role of neurogenins

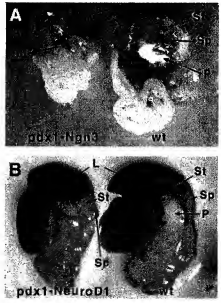

Fig. 8. Gross anatomy of the gut and associated organs of transgenic animals at E18.5 over expressing (A) ngn3 or (B) neuroD1 under the control of the *Pdx1* promoter. Non-transgenic littermates are shown at the right in each panel (wt). Arrows points to the following organs: liver (L), stomach (St), spleen (Sp), and pancreas (P). Reduced pancreatic tissue is not visible in the transgenic animals. Reductions in the stomach and small intestine are also visible in the transgenic animals.

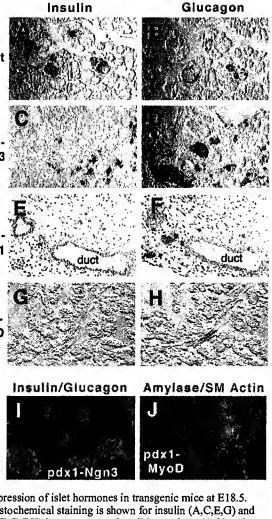

Fig. 9. Expression of islet hormones in transgenic mice at E18.5. Immunohistochemical staining is shown for insulin (A,C,E,G) and glucagon (B,D,F,H) in pancreases of a wild-type fetus (A,B) and transgenic fetuses expressing ngn3 (C,D), neuroD1/BETA2 (E,F) or MyoD (G,H) under the control of the *Pdx1* promoter. E and F show a single large duct with a small clump of remaining pancreatic tissue staining predominantly for glucagon. (I) Immunoflourescent staining for glucagon (cy3 label, orange) and insulin (fluorescein label, green) in the pancreas of a transgenic fetus expressing ngn3 at E18.5. (J) Immunoflourescent staining for smooth muscle actin (cy3 label, orange) and amylase (fluorescein label, green) in the pancreas of a transgenic fetus expressing MyoD, at E18.5. (200× magnification). Only pancreatic tissue is shown, with the exception of E and F which include surrounding loose connective tissue.

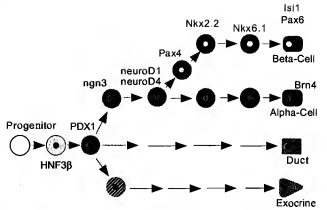

Fig. 10. Model for the role of ngn3 in endocrine differentiation in the mouse pancreas. The proposed position for each transcription factor is based on its timing of expression, timing of predominant functional role, or both. Clearly some factors function at several steps, but a single step is shown for simplicity.

in initiating neuroD expression during neural development (Lee, 1997; Ma et al., 1996). Although not the only possible explanation, the expression and transgenic studies are consistent with this model, as shown in Fig. 10. Recent studies of the mouse neuroD1 promoter also support the conclusion that neurogenin3 directly activates neuroD1 expression (Huang et al., 2000).

The transgenic animal experiments demonstrate that neurogenin3 and neuroD1/BETA2 drive endocrine differentiation; but the cell fate induced by ectopic ngn3 expression in these experiments is predominantly or exclusively α cells. Although it is possible that endogenous neurogenin3 only drives α cell differentiation, the co-expression of neurogenin3 with beta cell transcription factor Nkx6.1 during normal development strongly suggests the involvement of neurogenin3 in the differentiation of other pancreatic endocrine cells. In addition, the recent targeted disruption of the neurogenin3 gene in mice demonstrates that neurogenin3 is required for the formation of all four endocrine cell lineages (Gradwohl et al., 2000). Alternatively, the predominance of α cells in the transgenic animals could result from the abnormally early and broad expression of the transgenes under control of the *Pdx1* promoter. By E12.5 in the transgenic animals, the majority of cells in the pancreas have already differentiated, and the marked reduction in the size of the pancreas at E18.5 demonstrates that little further growth occurs. Normally in non-transgenic mice very few insulin-producing cells appear in the pancreas prior to E13, presumably due to the lack of factors such as Pax4 (Smith et al., 1999) that are required for β cell differentiation (Sosa-Pineda et al., 1997). These results suggest a model in which α cells are the default result of neurogenin3 expression, and additional signals are required to deviate neurogenin3-expressing progenitor cells to alternate cellular fates such as β cells (Fig. 10).

The ability to identify endocrine precursor cells based on neurogenin3 expression allows us to refine prior models of the cascade of transcription factors controlling islet cell differentiation (Edlund, 1998; Guz et al., 1995; Jensen et al., 2000; St-Onge et al., 1999). Combining our data with previous genetic and functional studies, we propose a model in Fig. 10 that places many of the key islet transcription factors on a map of islet cell differentiation. Rigorous genetic proof is lacking for some points on the map, but hopefully this proposed model will provide a basis for further studies and a frame work for future refinements.

It must be noted that our data is not in agreement with previous proposals that early cells expressing glucagon, PP or peptide YY function as precursors of other islet cells (Alpert et al., 1988; Herrera et al., 1994, 1991; Teitelman et al., 1993;

Upchurch et al., 1994). In the transgenic experiments the dramatically increased early pool of α cells does not result in the further differentiation of these cells to other islet cell types, even by E18.5. In the normal, non-transgenic animals, the absence of cells co-expressing multiple islet hormones during the peak of neurogenin3 expression and endocrine cell neogenesis (E15) suggests that islet cell type fate is determined prior to hormone expression. This conclusion is supported by the presence of the β cell transcription factor Nkx6.1 in some neurogenin3-expressing precursors. Nkx6.1 is never seen in glucagon-expressing cells (M. Sander and M. S. G., unpublished data; Oster et al., 1998), suggesting that these cells co-expressing neurogenin3 and Nkx6.1 are already determined to follow a β cell fate. We propose that both β cells and α cells independently derive from a non-hormone-expressing precursor that expresses neurogenin3, and islet cell type decisions are made prior to the expression of hormones.

This model does not address whether neurogenin3-expressing cells represent a sustained stem cell population, or a transient state through which individual progenitor cells progress. By definition, stem cells regenerate themselves while producing new differentiated cells. In contrast, the transgenic experiments show that neurogenin3 forces cells to differentiate and does not allow cells to remain in the undifferentiated state. This does not mean that there are no endocrine stem cells, but rather that if such cells exist, they do not express neurogenin3. The massive early differentiation of endocrine cells in the transgenic experiments demonstrates that prior to E13 most of the epithelial cells of the pancreas retain the capacity to differentiate into endocrine cells. The appearance of neurogenin3-expressing cells among the duct cells after E13 suggests that the ability to respond to neurogenin3 and to differentiate into endocrine cells persists in the duct cells after E13, and that duct cells may effectively function as endocrine stem cells.

Do duct cells in the mature pancreas retain the capacity to differentiate into endocrine cells? Experiments in pancreatic regeneration suggest that they may (Slack, 1995). If the adult duct cells can respond to the pro-endocrine bHLH genes, then in combination with other signals these genes potentially could be used to induce new β cell formation to replace the β cells lost to autoimmune destruction in individuals with type I diabetes.

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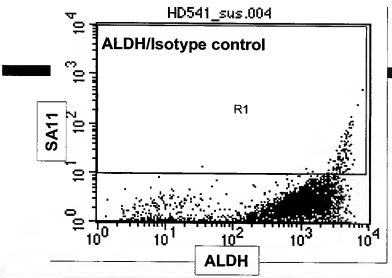
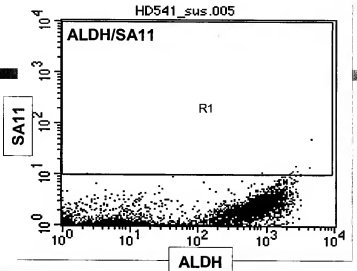
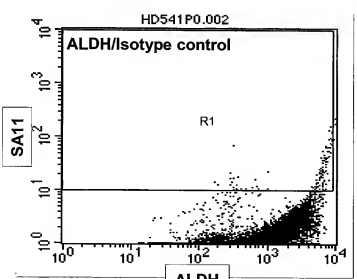
EXHIBIT E

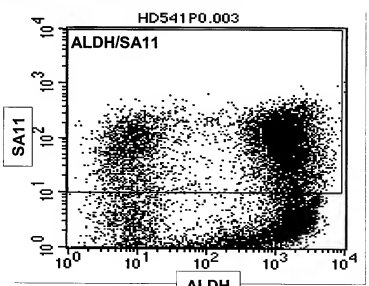
Percent SA11 Positivity in Non-plated HD541B P0 Cells





Percent SA11 Positivity in Plated HD541PP P0 Cells





Slide 1

SA11 is Undetectable in Non-plated HD541 Cells, but is Expressed Following Attachment and Culture

	Isotype	SA11
P0 non-plated	1.19%	0.12%
P0 plated and proliferated	1.31%	55.52%